

**IN THE SPECIFICATION**

Please amend the paragraph starting on page 7, line 3 and ending on page 7, line 6, as follows:

Saponins used as the adjuvants of the present invention are a group of compounds having a presenegenin skeleton, which belong to saponins having oleanane skeleton. The skeleton is indicated as olean-12-en-23,28-dioic acid,2,3,27-trihydroxy-(2 $\beta$ ,3 $\beta$ ,4 $\alpha$ ) in CAS nomenclature.

Please amend the paragraph starting on page 7, line 7 and ending on page 7, line 17, as follows:

Examples of saponins having the adjuvant activity and of which structures have been clarified include QS-21, etc. However, the inventive saponin is unambiguously different from QS-21 and derivatives thereof. QS-21 belongs to another group of saponins with oleanane skeleton, but its skeleton is quillaic acid to which a variety of sugars and others are linked. The quillaic acid skeleton is indicated as olean-12-en-28-oic acid,3,16-dihydroxy,23-oxo-(3 $\beta$ ,4 $\alpha$ ,16 $\alpha$ ) in CAS nomenclature. In other words, the presenegenin skeleton of saponins used as the inventive adjuvant is 23,28-dicarboxyl and has hydroxyl groups at positions 2, 3, and 27. On the other hand, the quillaic acid skeleton is 23-aldehyde-28-carboxyl and has hydroxyl groups at positions 3 and 16. The structures of the two are thus clearly different from each other.

Please amend the paragraph starting on page 11, line 4 and ending on page 11, line 20, as follows:

New vaccine preparations are provided by utilizing the inventive adjuvant. The vaccine preparations of the present invention include vaccines in both narrow and broad senses. Specifically, the vaccines include: i) vaccines interpreted as vaccines in a narrow sense, which are effective to infectious diseases of human and other animals caused by virus, bacterium, fungus, protozoan, other microorganisms, and vaccines interpreted in a broad sense. ~~Such~~ The vaccines in a narrow sense are exemplified by various vaccines such as influenza vaccine,

pertussis vaccine, purified pertussis-diphtheria-tetanus combined vaccine, Japanese encephalitis vaccine, hepatitis B vaccine, rotavirus vaccine, measles vaccine, rubella vaccine, mumps vaccine, measles-rubella-mumps combined vaccine, measles-rubella combined vaccine, and *Haemophilus influenzae* vaccine. The vaccines also include multi-drug resistant *Staphylococcus aureus* (MRSA) vaccine, *Helicobacter pylori* (abbreviated as *H. pylori* hereafter) vaccine, enterohaemorrhagic *Escherichia coli* (EHEC) vaccine, *Salmonella* vaccine, *Chlamydia* vaccine, *Mycoplasma* vaccine, AIDS vaccine, malaria vaccine, coccidium vaccine, and schistosome vaccine. Further, ~~ii—the~~ The vaccines in a broad sense are exemplified by vaccines, ~~which that~~ are effective in the prevention and treatment of non-infectious diseases, such as cancer vaccine, infertility vaccine, gastric ulcer vaccine, diabetic vaccine, and arteriosclerotic vaccine.

Please amend the paragraph starting on page 19, line 17 and ending on page 20, line 2, as follows:

Purified influenza viruses (A/PR/8/34 strain) were treated with ether to remove lipid constituents. The resulting HA vaccine (the amount of HA was 0.5 mg/ml) was mixed with the same volume of saline solution (1 mg/ml) of onjisaponins A, E, F, or G purified by the method as shown in Example 1 to give a vaccine preparation. The purity of each fraction of onjisaponin used was at least about 95% or higher. BALB/c mice (7-week-old females) were anesthetized with amobarbital. The vaccine preparation (20  $\mu$ l) was dropped into the left nasal cavity with a micro-pipette. After 4 weeks, whole blood was collected from the heart of each mouse under ether anesthesia to prepare the serum. The serum was first treated with RDE (receptor destroying enzyme) to remove nonspecific hemagglutinating substances. The serum was serially 2-fold diluted in U-shaped wells of a micro-titer plate. Each was mixed with 16 HA units of the virus. After the mixed sample was allowed to stand still for 30 minutes at room temperature, chicken erythrocytes were added thereto. The mixture was allowed to stand still at room temperature for an hour. Then, the titer of hemagglutination inhibiting (HI) antibody was evaluated.

Please amend the paragraph starting on page 20, line 13 and ending on page 21, line 15, as follows:

An influenza HA vaccine (1 mg/ml) prepared in the same manner as shown in Example 3 was mixed with the same volume of saline solution of the sample (1 mg/ml) to give a vaccine preparation. BALB/c mice (7-week-old females) were anesthetized by intraperitoneally inoculating sodium amobarbital. The vaccine preparation (20  $\mu$ l) was intranasally inoculated to mice. After bred for 3 weeks, the mice were further subjected to secondary intranasal inoculation of the vaccine alone. After the mice were bred for further one week, sera and nasal irrigation liquids were prepared from them. The titer of anti-influenza virus antibody in the serum was evaluated based on the HI antibody titer. After bloodletting, the nasal irrigation liquids were collected from the mice by perfusing the right and left nasal cavities twice with 1 ml of phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). The quantities of anti-HA-IgA and anti-HA-IgG antibodies in the nasal irrigation liquids were determined by enzyme linked immunosorbent assay (ELISA). Prior to the assay for anti-HA-IgA, each well of a 96-well EIA plate (Linbro) was treated with 100  $\mu$ l of HA vaccine (5  $\mu$ g/ml) suspended in a coating buffer (0.01 M carbonate-sodium bicarbonate buffer, pH 9.6). The plate was allowed to stand still for the coating at room temperature for 2 hours. The plate was then washed with Tween-20-containing PBS (abbreviated as PBS-Tween hereafter). Subsequently, each well was coated with 200  $\mu$ l of PBS containing 1% BSA and 0.1% NaN<sub>3</sub>. The plate was allowed to stand still at 4°C overnight, and then washed with PBS-Tween. Subsequently, a 100- $\mu$ l aliquot of protein G-Sepharose 4FF (Pharmacia-Biotech Co.) unadsorbed fraction of the nasal irrigation liquid was added to each well. The plate was allowed to stand still at room temperature for 2 hours and then washed with PBS-Tween. Subsequently, 100  $\mu$ l of alkaline phosphatase-labeled goat anti-mouse IgA ( $\alpha$ -chain specific, 1:1000) diluted with PBS containing BSA and 0.1% NaN<sub>3</sub> was added to each well. The mixture was allowed to stand still at room temperature overnight. Then, the plate was washed with PBS-Tween. Sodium *p*-nitrophenyl phosphate (1 mg/ml; Sigma Co.) dissolved in 10% diethanolamine buffer (pH 9.8) was added to each well. The mixture was allowed to stand still at room temperature for 20 to 30 minutes. Then, the color development was monitored with O.D. at 405 nm in a plate reader (type MRX-

MD, Dynex Co). The assay for anti-HA-IgG was carried out by using a protein G-Sepharose 4FF-adsorbed fraction of the nasal irrigation liquid as a sample and by using an alkaline phosphatase-labeled rabbit anti-IgG ( $\gamma$ -chain specific, 1:1000) as a secondary antibody.

Please amend the paragraph starting on page 22, line 9 and ending on page 22, line 20, as follows:

The saline solution (1 mg/ml) of onjisaponin used in Example 3 was mixed with a pertussis-diphtheria-tetanus combined vaccine (containing 15  $\mu$ g/ml of pertussis vaccine, 2 LF/ml of tetanus toxoid, 20 LF/ml of diphtheria toxoid, and 0.27 mg/ml of aluminum hydroxide gel; Kitasato Institute) to give a vaccine preparation. BALB/c mice (7-week-old females) were anesthetized by intraperitoneally inoculating sodium amobarbital. The vaccine preparation (20  $\mu$ l) was intranasally inoculated to each mouse. The same amount of the vaccine was further inoculated to each mouse after 4 weeks. The mice were bred for 2 weeks and then the sera and nasal irrigation liquids were collected from the mice. The evaluation of antibody titer was performed by ELISA for anti-pertussis toxin (PT)-IgG, anti-diphtheria toxoid (DT)-IgG, and anti-tetanus toxoid (TT)-IgG antibodies in the sera and for anti-PT-IgA, anti-DT-IgA, and anti-TT-IgA antibodies in the nasal irrigation liquids.

Please amend the paragraph starting on page 23, line 19 and ending on page 24, line 5, as follows:

It has been known that saponins generally have the hemolytic activity. Thus, the inventive active saponins were tested for the hemolytic activity as follows. The sheep erythrocytes were washed 3 times with phosphate buffered saline (PBS), and then diluted 2.5 times with the same buffer for the subsequent experiment. Aliquots (100  $\mu$ l) of solutions of onjisaponins A, E, F, and G (200, 100, 50, 25, 6.25, 3.125  $\mu$ g/ml-PBS solution) were added to V-shaped wells of a 96-well micro-plate, and 25  $\mu$ l of sheep erythrocyte suspension was added to each well. The plate was allowed to stand still at room temperature for 30 minutes. Then, the samples were centrifuged at 1000 rpm for 5 minutes. An aliquot (50  $\mu$ l) of the resulting supernatant was transferred to a flat-bottomed well of a plate and the absorbance thereof was

measured at 490 nm in a micro-plate reader (Model 450, BioRad Co.). The hemolytic activity of test compound was evaluated based on the increase in absorbance at 490 nm, which results from the release of hemoglobin from sheep erythrocytes. Figure 6 shows the hemolytic activities of onjisaponins A, E, F, and G. Large differences were found in the hemolytic activity among the compounds. The hemolysis was hardly recognized with onjisaponin E or F at a final concentration of 200  $\mu\text{g/ml}$ . On the other hand, a moderate degree of hemolysis was observed with onjisaponin G and hemolysis clearly occurred with onjisaponin A.

Please amend the paragraph starting on page 24, line 8 and ending on page 24, line 13, as follows:

Onjisaponins E and F were dissolved in PBS. The PBS solution was sterilized by filtration and then mixed with influenza HA vaccine (HA 1 mg/ml) so that 0.5 ml of the solution contained 5 to 10  $\mu\text{g}$  of the influenza HA vaccine and 10  $\mu\text{g}$  of the onjisaponins. The solution was added into containers, which was used as an influenza HA vaccine-onjisaponin injection. Such preparations can be stored at 10°C or lower in a cool and dark place.

Please amend the paragraph starting on page 24, line 21 and ending on page 24, line 28, as follows:

Onjisaponin F was dissolved in PBS and the solution was sterilized by filtration. The solution was mixed with a pertussis vaccine so that 20  $\mu\text{l}$  of the solution contained pertussis vaccine of which amount corresponded to 15  $\mu\text{g}$  of protein nitrogen and 10  $\mu\text{g}$  of the onjisaponin. A preservative (0.005% thimerosal) was added to the solution. The resulting mixture was added into containers, which was used as a pertussis vaccine-onjisaponin preparation to be used by intranasal inoculation. Such preparations should be stored at 10°C or lower in a cool and dark place.

Please amend the paragraph starting on page 25, line 12 and ending on page 25, line 18, as follows:

Onjisaponins F and G were dissolved in PBS and sterilized by filtration. The solution was mixed with a hepatitis B vaccine so that 1 ml of the solution contained HBs antigen of which amount corresponded to 40  $\mu\text{g}$  of protein and 10  $\mu\text{g}$  of the onjisaponin. A preservative (0.01% thimerosal) and stabilizer (0.2% porcine gelatin) was added to the solution. The resulting mixture was added into containers, which was used as a hepatitis B vaccine-onjisaponin injection. Such preparations should be stored at 10°C or lower in a cool and dark place.

Please amend the paragraph starting on page 25, line 26 and ending on page 26, line 2, as follows:

Onjisaponins F and G were dissolved in PBS. The PBS solution was sterilized by filtration and then mixed with Japanese encephalitis vaccine so that 1 ml of the solution contained inactivated Japanese encephalitis virus particles corresponding to  $10^{7.0}$  PFU and 10  $\mu\text{g}$  of onjisaponins. A stabilizer (0.2% porcine gelatin) was added to the solution. The resulting mixture was added into containers, which was used as a Japanese encephalitis vaccine-onjisaponin injection. Such preparations should be stored at 10°C or lower in a cool and dark place.

Please amend the paragraph starting on page 26, line 10 and ending on page 26, line 16, as follows:

Onjisaponin E was dissolved in PBS and the solution was sterilized by filtration. The solution was mixed with a measles vaccine so that 20  $\mu\text{l}$  of the solution contained measles vaccine of which amount corresponded to 20  $\mu\text{g}$  of the virus particles and 2.5  $\mu\text{g}$  of the onjisaponin. A stabilizer (0.2% porcine gelatin, 0.1% sodium glutamate, 5% lactose) was added to the solution. The resulting mixture was added into containers, which was used as a measles vaccine-onjisaponin nasal drop. Such preparations should be stored at 10°C or lower in a cool and dark place.

Please amend the paragraph starting on page 26, line 24 and ending on page 27, line 2, as follows:

Onjisaponin E was dissolved in PBS and the solution was sterilized by filtration. The solution was mixed with a rubella vaccine so that 20  $\mu$ l of the mixture contained rubella vaccine of which amount corresponded to 20  $\mu$ g of the virus particles, and 2.5  $\mu$ g of onjisaponin. A stabilizer (0.1% sodium glutamate, 5% lactose) was added to the solution. The resulting mixture was added into containers, which was used as a rubella vaccine-onjisaponin nasal drop. Such preparations should be stored at 10°C or lower in a cool and dark place.

Please amend the paragraph starting on page 27, line 10 and ending on page 27, line 16, as follows:

Onjisaponin E was dissolved in PBS and the solution was sterilized by filtration. The solution was mixed with a mumps vaccine so that 20  $\mu$ l of the solution contained mumps vaccine of which amount corresponded to 20  $\mu$ g of the virus particles and 2.5  $\mu$ g of the onjisaponin. A stabilizer (0.2% porcine gelatin, 0.1% sodium glutamate, 5% lactose) was added to the solution. The resulting mixture was added into containers, which was used as a mumps vaccine-onjisaponin nasal drop. Such preparations should be stored at 10°C or lower in a cool and dark place.

Please amend the paragraph starting on page 27, line 24 and ending on page 28, line 2, as follows:

Onjisaponin E was dissolved in PBS and the solution was sterilized by filtration. The solution was mixed with measles-rubella vaccine so that 20  $\mu$ l of the solution contained the respective vaccines of which amounts corresponded to 7  $\mu$ g of the virus particles and 2.5  $\mu$ g of the onjisaponin. A stabilizer (0.2% porcine gelatin, 0.1% sodium glutamate, 5% lactose) was added to the solution. The resulting mixture was added into containers, which was used as a measles-rubella vaccine-onjisaponin nasal drop. Such preparations should be stored at 10°C in a cool and dark place.

Please amend the paragraph starting on page 28, line 10 and ending on page 28, line 15, as follows:

Onjisaponins E and F were dissolved in PBS and the solution was sterilized by filtration. The solution was mixed with a rotavirus vaccine so that 20  $\mu$ l of the solution contained rotavirus vaccine of which amount corresponded to 3.3  $\mu$ g of the virus particles and 10  $\mu$ g of the onjisaponin. The solution was added into containers, which was used as a rotavirus vaccine-onjisaponin oral preparation or nasal drop. Such preparations should be stored at 10°C or lower in a cool and dark place.

Please amend the paragraph starting on page 28, line 24 and ending on page 29, line 2, as follows:

Onjisaponins E and F were dissolved in PBS and the solution was sterilized by filtration. The solution was mixed with a *Mycoplasma* vaccine so that 1 ml of the solution contained *Mycoplasma* vaccine of which amount corresponded to  $2.0 \times 10^{10}$  CFU (colony forming unit) of virus particles and 10  $\mu$ g of the onjisaponins. The solution was added into containers, which was used as a *Mycoplasma* vaccine-onjisaponin injection. Such preparations should be stored at 10°C or lower in a cool and dark place.

Please amend the paragraph starting on page 29, line 12 and ending on page 29, line 24, as follows:

An influenza HA vaccine (0.1 mg/ml) prepared from purified influenza viruses (A/Beijing/262/95 strain) in the same manner as shown in Example 3 was mixed with the same volume of saline solution of the sample (0.1 mg/ml) to give a vaccine preparation. BALB/c mice (7-week-old females) were anesthetized by intraperitoneally inoculating sodium amobarbital. The vaccine preparation (20  $\mu$ l) was intranasally inoculated to mice. After bred for 3 weeks, the mice were further subjected to secondary intranasal inoculation of a mixture of the vaccine and the sample. After the mice were bred for further one week, sera and nasal irrigation liquids were prepared from them. The titer of anti-influenza virus antibody in the serum was evaluated based on the HI antibody titer. After bloodletting, the nasal irrigation liquids were collected from the mice by perfusing the right and left nasal cavities twice with 1 ml of PBS containing 0.1% BSA.



The quantities of anti-HA-IgA and anti-HA-IgG antibodies in the nasal irrigation liquids were determined by ELISA.

Please amend the paragraph starting on page 29, line 25 and ending on page 29, line 30, as follows:

Figure 7 shows the influence of onjisaponin F on the production of anti-influenza virus antibody in the serum in secondary response. The onjisaponin F significantly increased HI antibody titer in the serum at a dose of 1  $\mu$ g per mouse as compared with the case where the HA vaccine alone was used. The adjuvant activity of onjisaponin F was slightly weaker than that of the same amount of CTB used as a positive control.

Please amend the paragraph starting on page 30, line 1 and ending on page 30, line 10, as follows:

Figure 8 shows the influence of onjisaponin F on the production of anti-influenza virus IgA and IgG antibodies in the nasal irrigation liquid in secondary response. The anti-HA-IgA and IgG antibodies were detected to be at low levels in the intranasal inoculation of the vaccine alone. On the other hand, onjisaponin F (1  $\mu$ g/mouse) significantly increased the anti-HA-IgA and IgG antibody titers in the nasal irrigation liquid in a group inoculated with the vaccine in combination with onjisaponin F. The adjuvant activity of enhancing the titer of anti-HA-IgA antibody was lower for onjisaponin F than for the same amount of CTB. However, onjisaponin F significantly exhibited the adjuvant activity of enhancing the titer of anti-HA-IgG, while CTB exhibited no adjuvant activity.